IMPACT: International Journal of Research in Engineering and Technology (IMPACT: IJREAT)
ISSN (P): 2347–4599; ISSN (E): 2321–8843
Vol. 8, Issue 10, Oct 2020, 7–18

BIOMARKER STUDIES FOR EARLY DETECTION OF CHRONIC KIDNEY DISEASE (CKD)

Parvathareddy Sudha Malini[1], S. Swathi[2] & Ethakota Jahnavi[3]

[1]Assistant Professor of OBG, Andhar Medical College, Visakhapatnam, Andhra Pradesh, India

[2]Research Scholar, M.Tech Biotechnology, J.N.T.U., Kakinada, Andhra Pradesh, India

[3]Research Scholar, Final Year MBBS, Siddhartha Medical College, Vijayawada, Andhra Pradesh, India

Received: 25 Sep 2020 **Accepted:** 09 Oct 2020 **Published:** 3 Oct 2020

ABSTRACT

Chronic Kidney Disease (CKD) represents a major challenge to public healthcare. Traditional clinical biomarkers of renal function (blood urea nitrogen and serum creatinine) are not sensitive or specific enough and only increase significantly after the presence of substantial CKD. Therefore, more sensitive biomarkers of CKD are needed. CKD-specific biomarkers at an early disease stage and early diagnosis of specific renal diseases would enable improved therapeutic treatment and reduce the personal and financial burdens.

The present study prompted to evaluate the presence and quantitation of the novel biomarker KIM-1 for early detection of chronic kidney disease. KIM-1 was detected in all the cohorts with chronic kidney disease. .

KEYWORDS: *Chronic Kidney Disease, Serum Creatinine, Kidney Injury Molecule, SDS-PAGE, Quantitative Real Time PCR*

INTRODUCTION

Research in the human physiology has achieved enhanced knowledge and understanding of diseases. Today treatment of diseases is able to save more lives than older days, however several areas in human physiology is still a mystery and needs more research. One of these areas is the renal diseases, which is a major health problem all over the world.

Chronic Kidney Disease

Chronic kidney disease (CKD) is a progressive loss in renal function over a period of months or years. The NKF has suggested the following definition of CKD: established kidney damage with structural or functional abnormalities or a glomerular filtration rate <60 ml/min/1.73 m^2 for three months or more. The classification of stages of CKD is based on the level of kidney function measured by GFR, where stage 1 represents kidney damage with normal or elevated GFR and stage 5 represents a GFR of less than 15 ml/min/1.73m^2 or who require treatment with dialysis.

Chronic kidney disease (CKD) is a worldwide health problem, affecting millions of people (Di Angelantonio *et al.*, 2007). The magnitude of the problem is poorly described by the number of people that will initiate renal replacement therapy (haemo dialysis, peritoneal dialysis and renal transplantation), as the incidence of 1- 3 per 10,000 per year in the general population may seem small (Lysaght, 2002; Dor *et al.*, 2007). However, chronic dialysis treatment and transplantation have an enormous impact on the life of individual patients and their families, and renal replacement

A common phenomenon in renal failure is progressive renal function loss irrespective of the underlying cause of the kidney disease.

The most common screening test for CKD is the measurement of serum creatinine. However, it as an insensitive measure, since as much as 50% of the nephron mass may be lost before creatinine concentration increases and levels are influenced by several factors such as sex, age, body mass, and diet. If an individual is obese there will be more muscle metabolism and a chance of release of abundant creatinine into the blood and urine (Parmar, 2002) thus it is an imperfect biomarker for the renal failure estimation and there is an urgent need for an early detection biomarker.

A recent study of nephrotoxicity in rodents showed that the sensitivity of SCr is poor, especially when histologic injury to the kidney is mild (Vaidya et al; 2010). Because of the limitations of SCr, there has been considerable interest recently in identifying a troponin for the kidney. Despite widespread acknowledgment of the limitations; definitions of CKD continue to rely on SCr as a diagnostic standard, perhaps because of the historical absence of validated primary biomarkers of injury. New biomarkers of tubular injury have been sought because the kidney tubule is the most metabolically active segment of the nephron and is uniquely susceptible to ischemic and nephrotoxic insults (Bonventure JV et al; 2009). Forty-six (37.4%) subjects developed CKD Network stage 1 CKD; 9 (7.3%) of whom progressed to stage 3. Preoperative KIM-1 and α-GST were able to predict the future development of stage 1 and stage 3 (Koyner 2010). Animal and human studies have resulted in a number of promising biomarkers that may revolutionize the diagnosis of CKD, enabling more accurate and earlier diagnosis of tubular injury, and clinical studies of these biomarkers in humans are increasing.

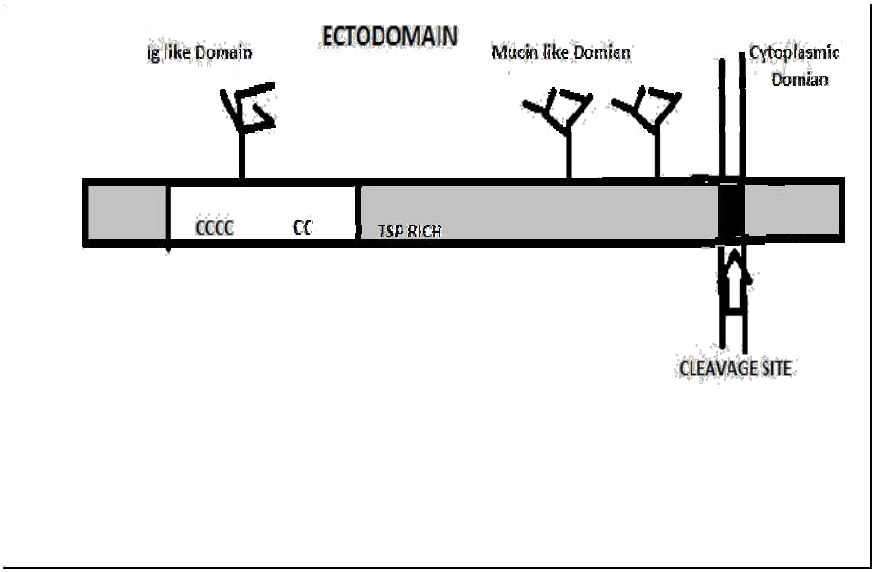

Figure 1

Kidney Injury Molecule-1

Kidney injury molecule-1 (KIM-1) is a type 1 membrane protein that is not expressed in normal kidney but is markedly up regulated in the injured proximal tubular epithelial cells of the human and rodent kidney in ischemic (Ichimura et al; 1998) and toxic (Ichimura et al; 2004) acute kidney injury. Kidney injury molecule-1 which is also referred as T cell

immunoglobulin and mucin domains – containing protein-1 (TIM-1) and hepatitis A virus cellular receptor-1 (HAVCR-1), is a type I transmembrane glycoprotein with an ectodomain containing a six cysteine immunoglobulin-like domain, two N – glycosylation sites, and a mucin domain. In an effort to identify molecules involved in kidney injury, the authors' laboratory originally discovered Kim-1 using representational difference analysis (a PCR-based technique) in rat models of acute ischemic kidney injury (Ichimura et al; 1998).

KIM-1, also expressed in other conditions where proximal tubules are dedifferentiated, including renal cell carcinoma, chronic cyclosporine nephrotoxicity (Perez et al; 2007) a protein-overload model of tubulointerstitial disease (Vaidya et al; 2006) and polycystic kidney disease (Kuehn et al; 2002). The KIM-1 family has eight members in mice, six in rats and three in humans (Kuchroo et al; 20

Structure of Human Kim-1:

The KIM-1b variant (referred to as KIM-1 hereafter) is mainly expressed by human kidney and contains two conserved tyrosine residues, including a predicted tyrosine kinase phosphorylation motif. The regulated shedding of KIM-1 ectodomain should contribute to regulation of ligand binding during the reparative response of the injured proximal tubule and potentially during the T_H2 immune response as well.

KIM-1 is shed constitutively into the culture medium of cell lines expressing endogenous or recombinant KIM-1 by membrane-proximal cleavage in a metalloproteinase-dependent manner after the proximal tubular kidney injury. Soluble KIM-1 is a very sensitive urinary biomarker of human tubular injury and is under investigation as a tissue and urinary biomarker of renal cell carcinoma. Despite the mounting evidence of its clinical utility, the regulation and underlying mechanism for KIM-1 cleavage are poorly characterized. KIM-1 shedding is enhanced dramatically by per vanadate, a potent inhibitor of protein tyrosine phosphatases.

MATERIALS AND METHODS

Urine and blood from the patients with diabetes and hypertension, or both or none was taken for the present study. The stage and the presence of chronic kidney disease were evaluated by the eGFR. Ten cohorts were selected for this study. Patients with different stages ranging from stage 1 to stage 5. The urine samples were subjected to SDS-PAGE. The bands resembling the molecular weight of the KIM-1 were then quantitated using real time-PCR.

Urine Sample Collection

Urine samples were collected from the patients of in plastic containers and immediately stored at -20^0C. The clinical characteristics of the cohorts was questioned and tabulated in the (Table 4). They were then used for further analysis of SDS- PAGE, RNA isolation and RT-PCR. Ten urine samples were collected for the current study out of which six are of patients suffering from CKD with different stages and another four are of the controls. The eGFR was calculated for each urine sample using the MDRD Equation and the stage of the patient was determined.

MDRD Equation

$GFR = 175 \times SerumCr^{-1.154} * age^{-0.203} * 1.212$ (if patient is black) * 0.742 (if female)

Table 1: Characteristics of Patients and Controls
Cohorts Subjected to SDS-PAGE Profiling and Quantitation Analysis

Sample	Gender	Age	DM	HTN	Serum Creatinine mg/dl	eGFR (ml/min)	CKD Stage
1	F	45	N	Y	0.7	90.4	1
2	M	28	N	N	3.5	21.0	3
3	M	50	Y	N	1.8	40.1	3
4	M	55	N	Y	4.6	13.3	4
5	M	35	N	Y	4.2	16.2	4
6	M	40	N	N	6.5	9.5	5
7	M	56	N	N	-	-	-
8	M	36	N	N	-	-	-
9	F	43	N	N	-	-	-
10	F	48	N	N	-	-	-

Molecular Analysis of Kim-1

The molecular analysis of KIM-1 was done by isolating the RNA from the urine samples of patients and controls. The samples which showed the RNA bands were considered for the further process. The RNA isolated was converted to cDNA using reverse transcription PCR and quantification of KIM-1 was done using real time PCR.

PROCEDURE

Day 1

The urine samples were ground in liquid nitrogen into 2ml Eppendorf tube. The extraction buffer was taken into a falcon tube and heated to 65°C in a water bath. Before it is used 2% (v/v) β-Mercaptoethanol was added and mixed well. About 600µl extraction buffer and 600µl phenol-chloroform isoamylalcohol were mixed thoroughly. The Phenol precipitates the proteins and chloroform precipitates the lipids.

Samples were incubated at 55°C for 10 min, and tubes were inverted from time to time and allowed for centrifugation at 11000rpm for 10 min at room temperature to separate the two phases.

The upper water phase which usually contains RNA was transferred into a fresh tube and re-extracted with 600µl phenol chloroform-isoamylalcohol. The tubes were mixed by inverting and centrifuged at 11000rpm for 10 min at 37°C. It is resolved into upper and lower phase.

The upper phase was transferred to the fresh tube and 200 µl 8M LiCl was added to each tube. This solution was kept overnight for the precipitation of RNA.

Day 2

The centrifuge tubes containing the precipitated RNA were cooled down to 4°C and spin down at same temperature at maximum speed for 20 min. Supernatant was removed with the pipette and the pellet was washed with 800µl 2M ice- cold lithium chloride in DEPC and the pellet was vortexed. Samples were spin down at 4°C at maximum speed for 10 min.

Supernatant was removed and washed pellet with 800µl ice-cold 80% ethyl alcohol in DEPC water. Again the samples were spin down at 4°C at maximum speed for 10 min and the supernatant was removed. This step was processed

once again and the excess ethyl alcohol was removed and pellet was subjected to air dry and dissolved in RNase free water. RNA concentration was measured using Spectrophotometer and integrity in gel.

c-DNA Synthesis from the Isolated RNA

Usually after we have extracted RNA from tissue we will synthesize cDNA from 500ug DNase treated RNA. 1µg of clean RNA was taken into each well in a 96 well PCR plate. Then a solution was prepared by mixing 10mM each dNTP Mix (10µl) and 100uM OligodT (10µl). This solution of 2µl was added to all the 10 samples and allowed to spin at 5min at 65 °C and returned to ice.

Master mix was prepared by adding 40µl of 5 X RT buffer, 40µl of 25m $MgCl_2$, 20µl of 0.1M DTT, 7.5µl of ambion-H_2O and 2.5µl of Super Script II (200U/µl). 11µl of the master mix is added to each sample and spin down at 42°C and 70°C for a period of 15 min and returned to ice. The converted c-DNA is now subjected for the qPCR.

Quantitative Real-Time PCR for KIM-1 Expression

The resultant cDNA was amplified separately with specific primers for Kim-1 5′ CGTCCAC CG CAAATGCTT - 3′ (forward) and 5'TCTGCGCAAGTTAGGTTTTGTC-3 (reverse) and β-actin. Amplification and detection were performed with the CFX96 Real Time PCR System (Bio-Rad) using emission from SYBR Green. After an initial activation step at 50°C for 2 min and a hot start at 95° C for 10 min., PCR cycles consisted of 40 cycles at 95°C for 15 sec. and 60°C for 60 sec. Specificity of qPCR products was routinely assessed by performing a dissociation curve. Gene expression was normalized with the mean of β-actin mRNA content and calculated relative to controls using the relative standard curve method. Results were finally expressed as 2^{-CT} (*C*T- threshold cycle). In brief, this method uses a single sample, termed the calibrator sample, as a comparator for every unknown sample's gene expression level. The calibrator can be any sample chosen to have all of the genes expressed. The calibrator was analyzed on every assay plate with the unknown samples of interest. The relative fold difference is calculated using the formula.

Fold induction = $2^{-\Delta\Delta C}{}_T$, where $-\Delta\Delta C_T$ (C_T of gene of interest in unknown sample -

C_T of β-actin gene in unknown sample) - (C_T of gene of interest in calibrator -C_T of β-actin gene in calibrator).

The fold differences in transcript abundance in samples from patients with different clinical phenotypes were compared.

RESULTS AND DISCUSSIONS

KIM-1 is a transmembrane protein (approx. 90KDa) which shed immediately after the ischemic injury of proximal tubule it was released into the urine. So electrophoretic analysis was performed for the visualization of the KIM-1 protein in the patients urine. It was run against a marker and the bands were visualized using gel doc and compared to a marker.

SDS-Profiling of Urine

Urine samples were collected from the in house patients and individuals with no signs of CKD were considered as controls. These urine samples were then subjected to SDS-PAGE. The present study suggested that proteins of different molecular weights have been resolved and they are low molecular weight proteins ranging between 35KDa-20KDa, middle molecular weight proteins ranging from 90KDa-45KDa and heavy molecular weight proteins ranging from 200KDa-95KDa.

Interestingly a protein of molecular weight 90KDa was visualized in all CKD patients including the stage 1 patient and was missing in the controls. Therefore it appears that 90KDa protein could be a reliable marker for identification of the disease.

In addition other proteins of molecular weight 66-35KDa also resolved in the patients and not in the controls but they showed a differential expression.. Patients with advanced stage are showing more protein content compared to an early stage individual. Thus the present study indicates that the quality and quantity of the protein is enhanced with progression of disease. Hence there is a direct co-relation between the amount of protein expressed and the stage of CKD. The gel electrophoresis pattern of each individual was seen in the (Figs 4.1, 4.2).

Many studies have suggest that urinary proteins like N-GAL and complement factors which are in the urine also have same molecular weight that of a KIM-

1(Sabbisetti,2013). So the present study had been extended for the confirmation and quantitation of KIM-1.

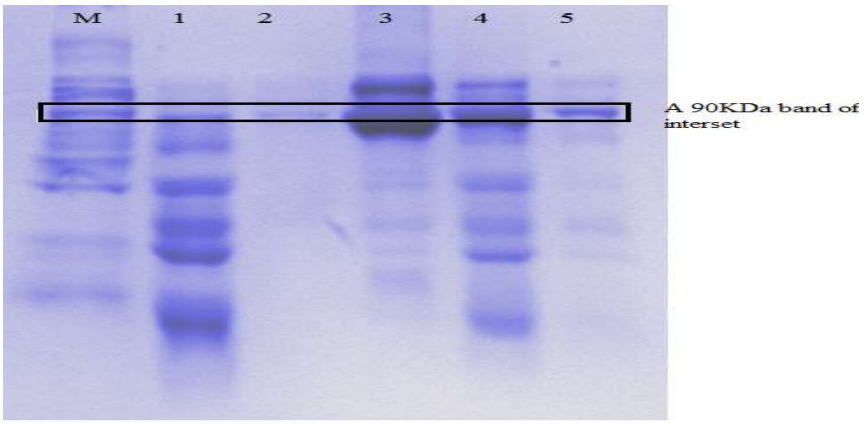

Figure 2: SDS-PAGE Electrophoretic Profiles of Urinary Proteins in the CKD Patients. Lane is Displayed as Follows. Lane M: Protein Marker. Lane 1: Sample from Patient with CKD Stage 5. Lane 2: Sample from Patient with Stage 4. Lane 3: Sample from Patient with CKD Stage 3. Lane 4: Sample from Patient with CKD Stage 2. Lane 5: Sample from Patient with CKD Stage 5.

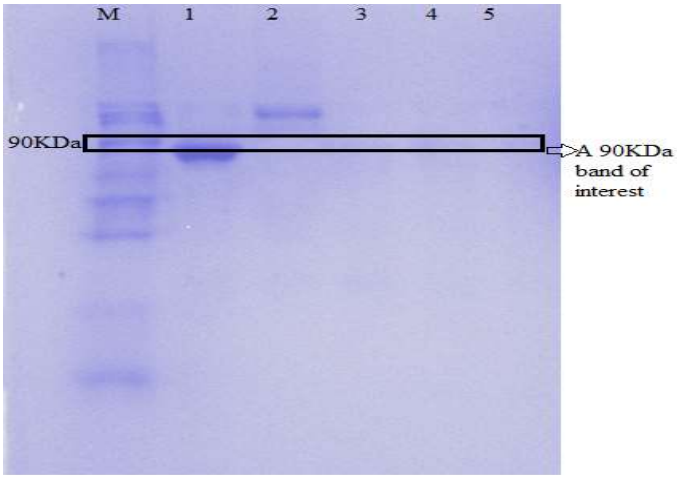

Figure 3: SDS-PAGE Electrophoretic Profiles of Urinary Proteins in the CKD Patients. Lane was Displayed as follows. Lane M: Protein Marker. Lane 1: Sample from Patient with CKD Stage 1. Lane 2-5: Samples from Controls.

RNA Isolation

RNA was isolated from the urine of patients and controls using lithium chloride method and run on an agarose gel for the visualization of a stable RNA profile. The CKD patients showed stable RNA profile but in controls it was not visualized. The integrity of the isolated RNA pattern is shown in (Fig 5)

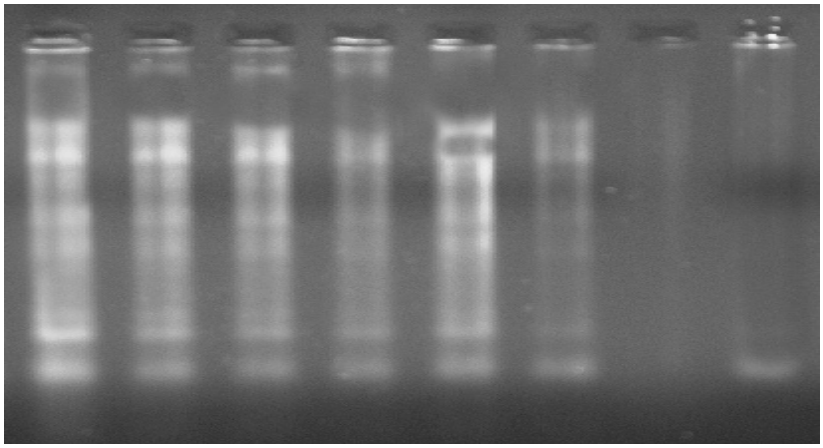

Figure 4: Lane 1 -6 Are Samples and Lane 7 and 8 are Negative Controls.

Quantitation of KIM-1 using qPCR

The isolated RNA was converted to c-DNA and was subjected to quantitative real time PCR for the quantitation of KIM-1 expression. Patients with undetectable urinary KIM-1 were excluded from the quantitative analysis and the samples included in the quantitative analysis were tabulated in the (Table 4). The relative transcript abundance of the shed urinary KIM-1 was compared against the stage of the chronic kidney disease and relation between the KIM-1 expression and age of the patient was also assessed. There is a differentiated expression of the KIM-1 gene in the same stage of renal failure and there is no association of KIM-1 expression with the creatinine levels. Thus the present study revealed that KIM-1 is sensitive marker for the progression of the disease and is a time-bound marker. It also showed that the KIM-1 expression is not associated with the creatine levels which assess the imperfectness of the traditional marker creatinine. The Gene expression was normalized with the mean of β-actin mRNA content and calculated relative to calibrator using the relative standard curve method.

Results were finally expressed as 2^{-CT} (*C*T threshold cycle) and were tabulated in (table 9, 10). The expression peaks of the KIM-1 with respect to CKD progression (fig 6.1) and age of the patient were seen in (fig 6.2).

Table 2: Quantitative PCR KIM-1 Expression Values with Respect to CKD Stage

Sample	CT	ΔΔCT	$_2$- *CT*
1	33.02	0	1
2	35.22	2.2	0.217638
3	33.31	0.29	0.817902
4	31.03	-1.99	3.97273
5	31.25	-1.77	3.41054
6	38.07	5.05	0.030186

Abundance Relative Transcriptive Abundance

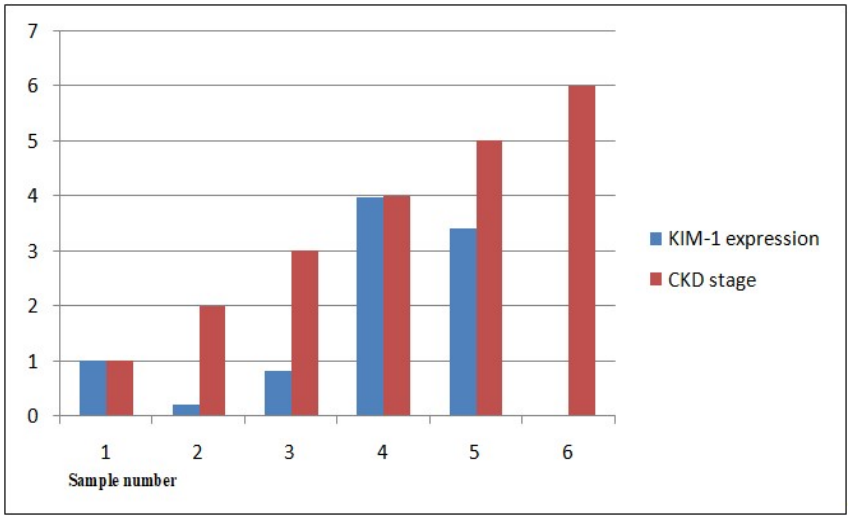

Figure 5: KIM-1 Gene Expression in the Human Urine Sample Related to CKD Stage.

Table 3: Quantitative PCR KIM-1 Expression Values with Respect to Age.

Sample	CT	ΔΔCT	$_2$- *CT*
1	33.02	-2.2	4.594793
2	35.22	0	1
3	33.31	-1.91	3.758091
4	31.03	-4.19	18.25222
5	31.25	-3.97	15.67072
6	38.07	2.85	0.138696

Relative Transcript Abundance

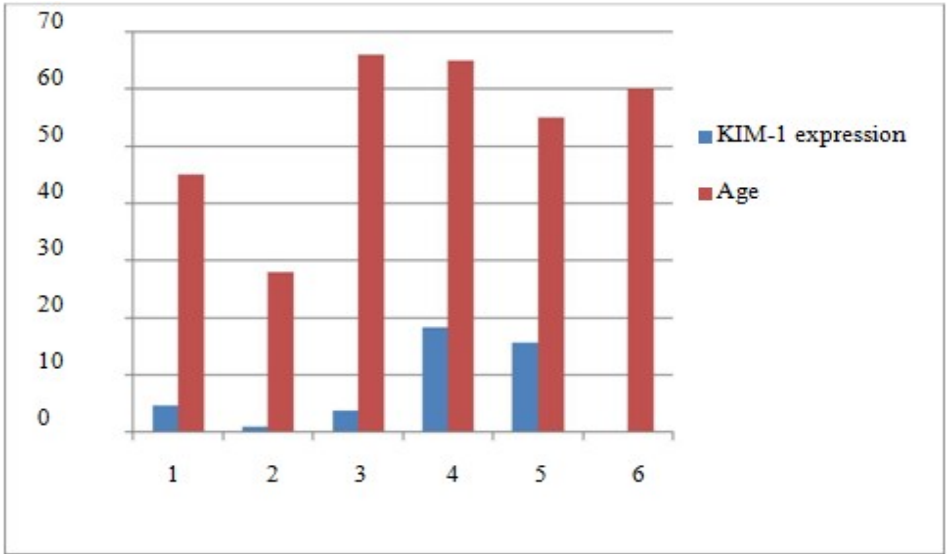

Figure 6: KIM-1 Gene Expression in the Human Urine Sample Related to Age.

DISCUSSIONS

The development of sensitive, specific, and early biomarkers for CKD will enhance the detection of the nephrotoxic potential of patients and compounds released into the urine. Better nephrotoxicity biomarkers will permit earlier diagnosis of CKD and allow medical personnel to intervene sooner to preserve residual renal function and improve health outcomes. In the present study, we put efforts to the expression of Kim-1, a new nephrotoxicity biomarker, represents a more sensitive and early biomarker of chronic kidney injury compared to traditional, routinely used biomarkers of nephrotoxicity in various pathological subject samples. The present project has setup for the identification and quantitation of KIM-1 expression in different CKD stage patients. The patients and the controls in the current study are nearly of the same age but differ in the clinical characteristics and the serum creatinine values. The eGFR values were calculated and compared to the staging and classification table. The patients with reduced eGFR for <15 were considered to be ESRD and the patients with near value of 90ml/min were considered to be early stage and were mainly focused in this study. The presence of middle to high molecular weight suggest glomerular lesions while increased low molecular weight protein may be a result of tubular lesion (Bazzi et al., 1997; Bazzi et al., 2000; Lee et al., 2005). The HMW (> 66 kDa) fractional clearance and albumin (66 kDa) fractional clearance were significantly correlated to the grade of glomerular lesion while the low molecular weight (< 66 kDa) fractional clearance was moderately correlated with the tubular lesions. In the present study three patients were having both glomerulo-tubular injuries whereas three patients are having only tubular injury. However this study showed higher tubular protein at 90KDa for all the patients but not detected in the controls. This may be KIM-1 which is seen only when there is damage in the tubules of nephrons. The actual size of the KIM-1 is 104KDa but only the ectodomain shed into the urine by the metalloproteases cleavage which is approximately 90KDa (Zhiwei, 2007). The other protein with molecular weight 66KDa may be mainly albumin.

In the present study two out of ten cohorts have albumin in their urine. The 74-76KDa band may be transferrin which is seen in the present study and is consistent with the previous study (Yalcin and Cetin, 2004). The 55-65KDa bands may be α1-antitrypsin while the 45 kDa was heavy chain IgG or IgA (Outteridge, 1985). The band between the 35-39KDa may be α_1-microglobulin. The protein at band 25-26 kDa may be retinol binding protein (22 kDa) or light chain IgG and IgA (25 kDa) (Outteridge, 1985). The lowest weight at 10-15 kDa may be a β2-microglobulin (12 kDa). The real time quantitative PCR showed variable levels of KIM-1 gene even at same stage of chronic kidney disease. This shows that KIM-1 is a reliable marker and expresses the severity and progression of the kidney failure. In the present study the KIM-1 expression levels in the stage 5 are less compared to stage 1, 2 and 3. These results are in accordance with the previous study that KIM-1 levels in urine were found to be elevated, but subsequently decreased when patients received treatment with angiotensin-converting enzyme inhibitors or a low-sodium diet. These findings suggest a potential role for KIM-1 as a measure of therapeutic efficacy (Waanders F et al; 2009). There is a gradual increase in the expression of KIM-1 in the patient with hypertension than without it that are in the same stage which correlates that hypertension is also a reason for the renal failure which leads to the impairment in the glomerular filtration (Keane, 1999). This revealed that clinical characteristics like diabetes and hypertension progresses the kidney failure and is directly proportional to the KIM-1 gene expression. The patient with stage 2 is having higher gene expression of which indicates that this patient is in high risk of CKD and progresses quickly to the ESRD. Thus the expression of this urinary marker reveals the injury at an early stage and indicated by the shedding of KIM-1 so it is considered to be as a reliable early injury marker. There also no association of KIM-1 gene expression with respect to age.

There is an increase in the KIM-1 expression of 0.8 times more in the patient with stage 1 who is having diabetes mellitus compared to a stage 2 individual. So it discloses that KIM-1 expression is sensitive and specific to the damage of the kidney progression.

CONCLUSIONS

In conclusion, the project has demonstrated that a soluble released form of human KIM-1 can be detected in the urine of patients with chronic kidney disease and may serve as a novel biomarker for renal proximal tubule injury. The expression of this biomarker is relative and proportional to the progression of the renal failure which indicates that this is a sensitive and specific biomarker. A larger prospective study is needed to further validate the utility of this biomarker in the clinical setting.

REFERENCES

1. *Bailly V, Zhang Z, Meier W, Cate R, Sanicola M, Bonventre JV. Shedding of kidney injury molecule-1, a putative adhesion protein involved in renal regeneration. J BiolChem 2002;277: 39739 –39748.*
2. *Ichimura T, Bonventre, JV et al. (1998). Kidney injury molecule-1 (KIM-1), a putative epithelial cell adhesion molecule containing a novel immunoglobulin domain, is up-regulated in renal cells after injury. Journal of Biological Chemistry1998;7: 4135-42.*
3. *Ichimura T, Hung CC et al (2004). Kidney injury molecule-1: A tissue and urinary biomarker for nephrotoxicant-induced renal injury. Am J Physiol Renal Physiology 2004; 286: 552–563.*
4. *Koyner JL, Vaidya VS et al. Urinary biomarkers in the clinical prognosis and early detection of acute kidney injury. Clin J Am Soc Nephrol 2010; 12: 2154-65.*
5. *Kuehn EW, Park KM, et al. Kidney injury molecule-1 expression in murine polycystic kidney disease.Am J Physiol Renal Physiol 2002; 283: 1326 -1336.*
6. *Nijboer, Damman et al. Kidney Injury Molecule-1 is an Early Noninvasive Indicator for Donor Brain Death-Induced Injury Prior to Kidney Transplantation. Am JTransplant 2009;8: 1752-1759.*
7. *Prozoileck WC, Edwards JR et al. Expression of kidney injury molecule-1 (KIM-1) in relation to necrosis and apoptosis during the early stages of Cd-induced proximal tubule injury.Toxicol Appl Pharmacology 2009; 238:306-14.*
8. *Remuzzi G., Bertani T. Pathophysiology of progressive nephropathies.N Engl J Med 1998; 339: 1448-1456.*
9. *Sabbisetti V, Humphreys BD. Chronic epithelial kidney injury molecule-1 expression causes murine kidney fibrosis. J Clin Invest 2013; 9: 4023-35*
10. *Sabbisetti VS, Waikar SS et al. Blood kidney injury molecule-1 is a biomarker of acute and chronic kidney injury and predicts progression to ESRD in type I diabetes. JAm Soc Nephrol. 2014;10:2177-86.*
11. *Sushrut S, Waiker et al. Imperfect Gold Standards for Kidney Injury Biomarker Evaluation. J Am Soc Nephrol 2012; 1: 13–21.*

12. *Vaidya VS, Bailly V et al. Tubular kidney injury molecule-1 in protein-overload nephropathy. Am J Physiol Renal Physiol 2006; 291: 456 –464.*
13. *Vaidya VS, Ozer JS et al. Kidney injury molecule-1 outperforms traditional biomarkers of kidney injury in preclinical biomarker qualification studies. NatBiotechnology 2010;28: 478–485.*
14. *Zhiwei et al. Shedding of the Urinary Biomarker Kidney Injury Molecule-1 (KIM-1)Is Regulated by MAP Kinases and Juxtamembrane Region. Journal of Americansociety of nephrology 2007;10: 2704-2714.*